T-cell development: **A new marker of differentiation state**

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Differentiation of T cells is a complicated affair and there has been a dearth of markers that faithfully reflect thymocyte phenotype. A new strategy based on T-cell receptor gene sequencing has revealed a marker that can be used to monitor thymocyte differentiation with fidelity and without perturbation.

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T-lymphocyte differentiation occurs primarily in the thymus (reviewed in [1]), which is seeded by poorly characterized T-cell progenitors that are unreactive to foreign antigens. From the thymus emerge mature T cells that, collectively, are capable of responding to a multitude of antigens — recognized by the antigen-specific T-cell receptor as peptides bound to major histocompatibility complex (MHC) molecules. In between these two developmental stages, an elaborate program of differentiation unfolds, accompanied by massive proliferation and death, such that millions of cells are produced within the thymus, but only a small fraction emigrates into the peripheral circulation.

Pathways of thymocyte differentiation have generally been traced by following the display of particular cell-surface proteins. Chief amongst these are the CD4 and CD8 co-receptor molecules, whose pattern of expression defines a progression of thymocyte differentiation in three stages: $CD4^-CD8^-$ cells include the most immature T-cell progenitors; these differentiate into $CD4^+CD8^+$ cells, which constitute the bulk of thymocytes and are the first to express the αβ T-cell receptor; fully mature $CD4^+CD8^-$ or $CD4^-CD8^+$ cells display the αβ T-cell receptor at high levels, proliferate in response to antigen stimulation, and exhibit either of the characteristic helper or cytotoxic effector functions of T cells. This three-stage pathway has been embellished to an almost baroque degree by further detailing expression levels of the two co-receptors or by monitoring the display of other cell-surface proteins. Nevertheless, this pathway remains a useful simplification, allowing the major events in the life of a thymocyte to be situated easily.

Two critical events coincide with the transition of thymocytes from being $CD4^+CD8^+$ to $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. In a process termed positive selection, the repertoire of T cells is enriched for those capable of recognizing foreign antigens in association with self-expressed MHC molecules. In addition, cells must decide whether to become $CD4^+$ helper T cells which recognize antigens in association with MHC class II molecules or $CD8^+$ cytotoxic cells reactive to antigens bound to MHC class I complexes, a process called lineage commitment. It is generally agreed that the key to both of these events is the interaction between αβ T-cell receptors expressed on a differentiating thymocyte and MHC molecules displayed on thymic stromal cells [2].

Beyond this agreement, however, controversy reigns. There is no consensus on exactly which route a $CD4^+CD8^+$ cell follows to become a $CD4^+CD8^-$ or $CD4^-CD8^+$ cell, or precisely when positive selection or lineage commitment takes place, or what mechanisms are involved in executing these events [3]. At the root of many of the disagreements is the inadequacy of existing differentiation markers — in particular, their inability to trace transition events occurring over a timescale that is short compared with the turnover of surface proteins. For example, surface levels of CD4, CD8 and the αβ T-cell receptor have generally been taken as indicators of positive selection and/or lineage commitment, but expression of these molecules at the cell surface is far removed from the transcriptional programs underlying differentiative changes, and too much reliance on this parameter has prompted faulty conclusions to be drawn more than once in the past. Also, most attempts to experimentally verify the differentiation status of a cell suggested by its surface marker profile have involved purifications based on antibody engagement of the same markers, a process that could easily disturb normal maturation.

A way around this difficult problem has recently been reported by Sant'Angelo *et al.* [4], who have used T-cell receptor sequences as a marker of thymocyte differentiation state. To reduce the heterogeneity of the T-cell receptor repertoire, they began with a transgenic mouse line that carries a functionally rearranged gene encoding the T-cell receptor β chain, which was derived from a T-cell hybridoma specific for a peptide of myelin basic protein presented by MHC class II A^u molecules. The T-cell receptor α chain expressed by the hybridoma cells was encoded by a rearranged gene with the juxtaposed variable (V) and joining (J) segments Vα2.3 and Jα11. Thus, the authors focused on the sequence of the randomly generated junctional regions of Vα2.3–Jα11 transcripts in the T-cell receptor β transgenic mouse line.

Both $CD4^+CD8^-$ thymocytes and peripheral $CD4^+$ lymphocytes showed a striking enrichment for the precise junctional region sequence found in the T-cell receptor α gene sequence of the hybridoma cells; in addition, these two populations were depleted of out-of-frame junctional sequences. There were no such sequence enrichments and depletions in the Vα2.3–Jα11 junctional regions of immature $CD4^+CD8^+TCR^{lo}$ thymocytes, nor in $CD4^-CD8^+$ thymocytes or peripheral $CD8^+$ lymphocytes. The most likely explanation therefore is that, in the presence of the appropriate MHC molecules, in this case MHC II A^u, cells expressing the transgene-encoded Vβ chain together with the 'parental' Vα chain are efficiently positively selected into the CD4 lineage. Thus, the authors have taken the frequency of this particular junctional region in this T-cell receptor β transgenic mouse line as a marker for positive selection mediated by MHC class II molecules and as a marker for CD4 or CD8 lineage commitment.

Sant'Angelo *et al.* [4] have used this marker to make three points. First, they regarded their observation of the striking enrichment of a particular Vα–Jα junctional segment during positive selection to be an indication of the important role of thymic peptides in this process. Their reasoning was that X-ray crystallographic studies have repeatedly demonstrated that the region of the T-cell receptor α chain specified by the V–J junctional segment is in intimate contact with the peptide component of the MHC–peptide ligand [5]. A previous report from this group used a similar approach to make the same point about the importance of thymic peptides, although this earlier study relied on T-cell receptor β transgenic mice that had an artificially restricted complement of thymic peptides [6]. The role of peptides in positive selection has also been established by a number of other investigators using a variety of strategies [7].

Second, the authors exploited the difference between $CD4^+CD8^-$ and $CD4^-CD8^+$ cells to chart the sometimes tortuous thymocyte differentiation pathways. They took a high frequency of the parental Vα2.3–Jα11 junction to be a marker of the $CD4^+$ lineage and a low frequency to be indicative of the $CD8^+$ lineage. It should be kept in mind that cells yet to undergo positive selection, as well as $CD8^+$ cells, have the low-frequency phenotype, however, and therefore lineage assignment may not always be quite so straightforward. Their results were consistent with a previously published scheme [8], and highlighted the fact that surface levels of CD4 and CD8 can be poor indicators of lineage commitment, as pointed out by others [9,10].

Third, Sant'Angelo *et al.* [4] made use of the difference between $CD4^+CD8^+TCR^{lo}$ and $CD4^+CD8^-TCR^{hi}$ thymocytes to determine when positive selection is first evident. For this, they examined the various intermediate populations defined on the basis of thymocyte size — which varies according to proliferation and activation state — and levels of CD4, CD8, TCR and CD69 (an early T-cell activation marker closely associated with positive selection). It was somewhat surprising to find signs of positive selection already in $CD69^-$, large, $CD4^+CD8^+$ cells, yet this observation is consistent with an earlier report indicating that inducibility of CD69 gene expression, rather than CD69 cell-surface expression, is an early marker of positive selection [11]. So it seems that positive selection happens rather early during the 3–4-day life span of a $CD4^+CD8^+$ cell. It is noteworthy, however, that the enrichment for parental junctional sequences at this early $CD4^+CD8^+$ stage was far less striking than at the fully mature $CD4^+CD8^-$ stage, suggesting that the selection process continues for days.

We have recently reported a second strategy for side-stepping the problems with existing thymocyte differentiation markers [12]. Using homologous recombination in embryonic stem cells, we replaced the coding sequences of one of the copies of the CD4 gene with a β-galactosidase gene, thereby rendering β-galactosidase a faithful reporter of CD4 gene activity. It was also a more immediate indicator of CD4 gene activity than cell-surface CD4 levels, being cytoplasmic and having a faster turnover rate than CD4 itself. Thus, β-galactosidase expression could be used to distinguish cells committed to the $CD4^+$ or the $CD8^+$ lineage independent of surface levels of the two co-receptors. This new marker was also useful in tracing pathways of thymocyte differentiation and in re-examining some of the controversial issues related to CD4 and CD8 lineage commitment.

Hopefully, the judicious use of markers such as those described here, used singly or in combination, will stimulate progress in resolving still-contentious points related to thymocyte positive selection and lineage commitment. Certainly, it's about time we moved forward on these issues.

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